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Broad-spectrum non-nucleoside inhibitors for caliciviruses

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ABSTRACT

Viruses of the *Caliciviridae* cause significant and sometimes lethal diseases, however despite substantial research efforts, specific antivirals are lacking. Broad-spectrum antivirals could combat multiple viral pathogens, offering a rapid solution when no therapies exist. The RNA-dependent RNA polymerase (RdRp) is an attractive antiviral target as it is essential for viral replication and lacks mammalian homologs. To focus the search for pan-*Caliciviridae* antivirals, the RdRp was probed with non-nucleoside inhibitors (NNIs) developed against hepatitis C virus (HCV) to reveal both allosteric ligands for structure-activity relationship enhancement, and highly-conserved RdRp pockets for antiviral targeting. The ability of HCV NNIs to inhibit calicivirus RdRp activities was assessed using *in vitro* enzyme and murine norovirus cell culture assays. Results revealed that three NNIs which bound the HCV RdRp Thumb I (TI) site also inhibited transcriptional activities of six RdRps spanning the *Norovirus*, *Sapovirus* and *Lagovirus* genera of the *Caliciviridae*. These NNIs included JTK-109 (RdRp inhibition range: IC_{50} 4.3 −16.6 μM), TMC-647055 (IC_{50} range: 18.8−45.4 μM) and Beclabuvir (IC_{50} range: 23.8−>100 μM). *In silico* studies and site-directed mutagenesis indicated the JTK-109 binding site was within the calicivirus RdRp thumb domain, in a pocket termed Site-B, which is highly-conserved within all calicivirus RdRps. Additionally, RdRp inhibition assays revealed that JTK-109 was antagonistic with the previously reported RdRp inhibitor pyridoxal-5′-phosphate-6-(2′-naphthylazo-6′-nitro-4′,8′-disulfonate) tetrasodium salt (PPNDS), that also binds to Site-B. Moreover, like JTK-109, PPNDS was also a potent inhibitor of polymerases from six viruses spanning the three *Caliciviridae* genera tested (IC_{50} range: 0.1−2.3 μM). Together, this study demonstrates the potential for *de novo* development of broad-spectrum antivirals that target the highly-conserved RdRp thumb pocket, Site-B. We also revealed three broad-spectrum HCV NNIs that could be used as antiviral scaffolds for further development against caliciviruses and other viruses.

Abbreviations: BS-AV, broad-spectrum antivirals; CI, confidence intervals; FCV, feline calicivirus; IC_{50}, half maximal inhibitory concentration; HBV, hepatitis B virus; HCV, hepatitis C virus; HIV, human immunodeficiency virus; MNV, murine norovirus; NA, nucleoside analogue; NAF2, naphthalene di-sulfonate; NoV, norovirus; NNI, non-nucleoside inhibitor; NTP, nucleoside triphosphate; Pβ, Palm β; PDB, Protein Data Bank; PI, Palm I; PII, Palm II; PFU, plaque forming units; PPNDS, pyridoxal-5′-phosphate-6-(2′-naphthylazo-6′-nitro-4′,8′-disulfonate) tetrasodium salt; RCV, rabbit calicivirus; RdRp, RNA-dependent RNA polymerase; RHDV, rabbit hemorrhagic disease virus; SaV, sapovirus; TI, Thumb I; TII, Thumb II.

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1. Introduction

There is currently a lack of both narrow and broad-spectrum antivirals (BS-AVs) available to combat viral pathogens (Debing et al., 2015). This deficiency of BS-AVs is due to the complexity of viral replication strategies and their various host tropisms (Martinez et al., 2015). Antivirals are generally targeted against a single viral species, for example: hepatitis B virus (HBV), hepatitis C virus (HCV), herpesviruses or human immunodeficiency virus (HIV) (Gotte and Feld, 2016; Murakami et al., 2015; Cihlar and Fordyce, 2016), and cross-species antiviral activity is rare. BS-AVs could be used to combat several clinically significant and emerging viral pathogens when no specific therapies exist (Debing et al., 2015). Additionally, they could be used as prophylaxis in an outbreak setting, to treat chronically infected patients, or when rapid and accurate diagnoses are not feasible (Warren et al., 2014). Given the global health burden created by caliciviruses, there is a necessity to develop safe and effective BS-AVs against all *Caliciviridae* pathogens.

One strategy in BS-AV development is to target conserved domains of viral enzymes critical for replication (Debing et al., 2015). The viral RNA-dependent RNA polymerase (RdRp) is an attractive antiviral target, given its highly-conserved structure across viral families. RdRps are essential for viral replication and largely lack host homologs, minimizing chances of off-target effects (Malet et al., 2008). Despite significant sequence diversity, all RdRps form structural homomorphs resembling a closed right-hand with fingers, palm and thumb domains (Ng et al., 2008; Ferrer-Orta et al., 2006). To identify broadly conserved RdRp regions for further antiviral targeting, bioactive molecules can be identified by screening compounds or libraries using polymerase assays with RdRps from several viral species (Gong et al., 2013). This approach can reveal both highly-conserved viral enzyme domains, and BS-AV candidates with activity against different species, genera and families.

Caliciviruses are a diverse family of positive-sense RNA viruses, currently classified into five genera; *Norovirus, Sapovirus, Lagovirus*, *Nebovirus* and *Vesivirus* (Clarke et al., 2012). Viruses from the *Caliciviridae* cause significant diseases in a wide range of vertebrate hosts (Clarke and Lambden, 1997), and the substantial impact caused by caliciviruses is often underestimated (van Asten et al., 2011; Lee et al., 2012; Delibes-Mateos et al., 2014). Currently there are no specific antivirals available for the treatment of calicivirus infections, and vaccines are only available for two viruses, feline calicivirus (FCV) and rabbit hemorrhagic disease virus (RHDV) (Radford et al., 2006; Abrantes et al., 2012).

Among the caliciviruses that infect humans, norovirus (NoV) is a leading cause of acute gastroenteritis (Koo et al., 2010; Ahmed et al., 2014), accounting for approximately 219,000 deaths annually (Bartsch et al., 2016). Antiviral development faces significant challenges, partly because a cell culture system to propagate human NoV was only recently established (Jones et al., 2014; Ettayebi et al., 2016). Consequently, much of what is known about human NoV biology has been inferred from other caliciviruses including FCV and murine norovirus (MNV) (Karst et al., 2003). Both viruses are easily cultured, and MNV in particular has been widely used to screen potential human NoV antivirals in cell culture and mouse models (Wobus and Thackray, 2006).

Other significant *Caliciviridae* pathogens include sapovirus (SaV), FCV, and RHDV. SaV infects a wide range of species including humans, swine and marine mammals (Tse et al., 2012), whilst RHDV is a highly contagious pathogen that has been used as an effective rabbit biocontrol agent (Green et al., 2000). Together with benign rabbit calicivirus (RCV), RHDV is a member of the *Lagovirus* genus that infects the European rabbit, *Oryctolagus cuniculus* (Capucci et al., 1996).

Non-nucleoside inhibitors (NNIs) are antivirals that bind to the RdRp allosterically, preventing polymerase conformational changes required for replication (Caillet-Saguy et al., 2011). All clinically approved NNIs to-date offer only narrow-spectrum, species-specific activity, developed against either HIV (US Food and Drug Administration, 2014) or HCV (Hepatitis C Support Project, 2016). The HCV RdRp has five defined NNI binding sites (Eltahla et al., 2015) including: Thumb I (TI), Thumb II (TII), Palm I (PI), Palm II (PII) and Palm β (Pβ) (Fig. 1A). Previously, we reported that HCV TI- and PII-binding NNIs exhibit cross-genotypic activity against HCV (Eltahla et al., 2014a), indicating the potential for a wider antiviral spectrum. To identify broad-spectrum calicivirus RdRp targets, we used HCV NNIs as an initial probe for inhibitory activity against the human NoV RdRp, and then against a range of caliciviral RdRps.

Whilst HCV NNI RdRp binding sites are well defined, much less is known about the human NoV RdRp. Currently only three human

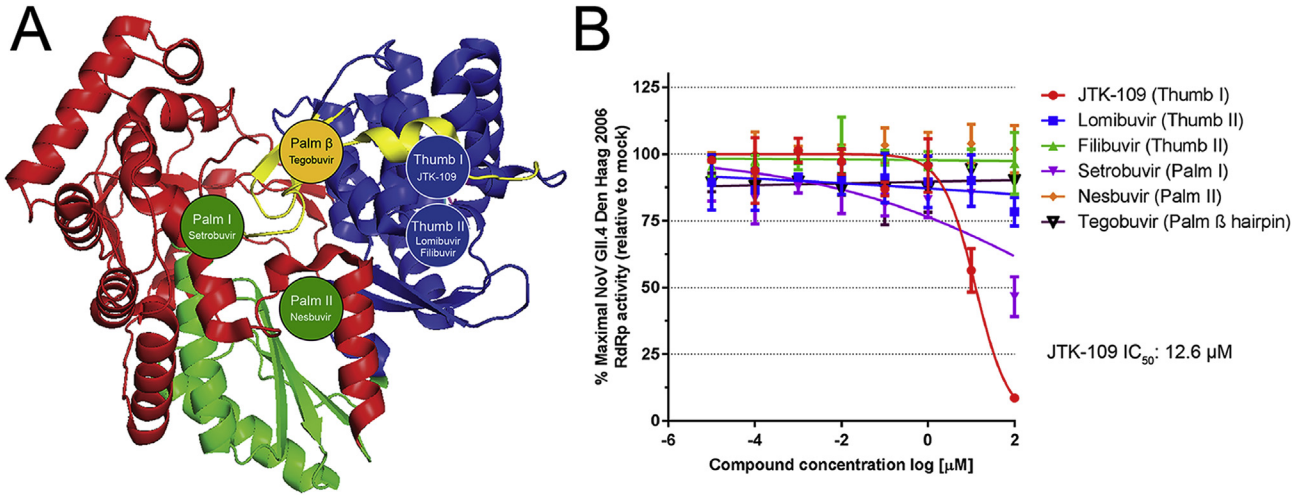

Fig. 1. Probing the human NoV RdRp for cross-inhibitory activity with HCV NNIs. A) The HCV RdRp domains are color-coded on the ribbon diagram: fingers (red), palm (green), thumb (blue) and palm β (yellow). HCV NNI binding sites are shown in colored circles, adapted from (Eltahla et al., 2015). B) Inhibitory effects of six HCV NNIs on human NoV GII.4 Den Haag 2006b RdRp activity were examined using a fluorescence-based RdRp transcription assay. NNIs (0.01 pM–100 μM) were compared to samples containing the compound vehicle only (0.5% DMSO [vol/vol]). Mean values from triplicate technical replicates are plotted with standard deviations.